

REMARKS

Claims 106-108, 115, 117-118, 120-133 and 135-143 were pending in the instant application. Claims 106-108, 115, 126, 129, 132, 133, 135, and 138-143 have been amended, and claims 127, 128, 130, 131, 134 and 136 have been cancelled. Accordingly, claims 106-108, 115, 117-118, 120-126, 129, 132-133, 135 and 137-143 will be pending in the application upon entry of the instant amendment. No new matter has been added.

Any amendments to and/or cancellation of the claims was done solely to more particularly point out and distinctly claim the subject matter of Applicants' invention to expedite the prosecution of the application. Applicants reserve the right to pursue the claims as originally filed in this or in a separate application(s). Support for the amendments to the claims can be found throughout the specification and claims as originally filed. Specifically, support for the amendments to claims 107, 108, 135, 138 and 141 can be found, for example, at page 23, lines 9-11 of the specification.

Rejection of Claims 107-108, 115, 117-118, 120-124, 129, 131-133 and 136***Under 35 USC 112, First Paragraph***

The Examiner has rejected claims 107-108, 115, 117-118, 120-124, 129, 131-133 and 136 under 35 USC 112, first paragraph, as "containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed had possession of the claimed invention." The Examiner states that, "[i]f Applicant were to insert the functional activity into the claim, then this rejection would be withdrawn."

In the interest of expediting prosecution, and in no way acquiescing to the Examiner's rejection, Applicants have amended claims 107, 108, 135, 138 and 141 to indicate the MRP-β polypeptide functions to transport, expel, or sequester substances from an intracellular milieu, and have cancelled claims 130, and 131. It is believed the present rejections render the rejection moot.

Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw the foregoing rejection.

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Rejection of Claims 106-108, 115, 117-118, 120-133, 129 and 135-143***Under 35 USC 112, First Paragraph***

The Examiner has rejected claims 106-108, 115, 117-118, 120-133, 129 and 135-143 under 35 USC 112, first paragraph, because, “the specification, while being enabling for identifying drugs which inhibit MRP-β by testing for MRP-β expression, for cell survival, or for sequestration of a cytotoxin, does not reasonably provide enablement for identifying stimulatory modulators of MRP-β, does not reasonably provide enablement for the scope of the modulators of claim 132, and does not reasonable provide enablement for identifying modulators by testing for sequestration of substrates other than cytotoxins.”

Solely in the interest of expediting prosecution, and in no way acquiescing to the Examiner’s rejections, Applicants have cancelled claims 127, 128, 130 and 131 thereby rendering this rejection, as it pertains to these claims, moot.

Applicants have amended claims 106-108, 129, 132, 133, 135, and 138-143 to be directed inhibitors, rather than modulators, of MRP-β.

The Examiner has also rejected claim 132 indicating that the specification does not provide enablement for the full scope of the inhibitors claimed. Applicants respectfully point out support for limitations in claim 132 are disclosed in the specification at, for example, at page 21, lines 16-20. Moreover, these compounds are well known by the ordinary skilled artisan. Furthermore, Applicants provide broad disclosure of methods of assaying activities for which any compounds to be tested (see, for example, page 50, line 1 through page 51, line 2. Therefore, one of ordinary skill in the art would find claim 132 to be fully enabled, and would be able to test these compounds for inhibitory activity as recited in the methods of claims 106-108 using only routine experimentation.

Lastly, the Examiner has rejected the foregoing claims because the specification, “does not reasonably provide enablement for identifying modulators by testing for sequestration of substrates other than cytotoxins.” Applicants respectfully traverse this rejection. Applicants respectfully submit that the ordinary skilled artisan would be able to practice the claimed methods using the teachings provided in the specification and the

knowledge available to one of skill in the art. For example, the specification teaches assays that one of skill in the art could use to test for the ability of MRP- β to transport substrates including, but not limited to, cytotoxins (see, for example, the assays taught on page 50-51 of the specification, and the description presented on pages 42 and 44). In Example 5, Applicants teach that only routine confirmatory testing is required to assess examples of additional substrates beyond cytotoxins.

Moreover, Applicants teach that MRP- β is a member of the ABC transporter family of proteins. The ABC transporter family has been shown to transport a number of substrates, and therefore one of skill in the art would have a large body of literature available from which they could identify potential substrates to use in the claimed assays. For the Examiner's convenience Higgins, C.F. ((1992) Annu. Rev. Cell. Biol. 8:67-113) is submitted herewith as Appendix A. Higgins, cited on page 3 of the instant specification, teaches that, as of 1992, the ABC transporter family was known to transport substrates that include, for example, vitamins, amino acids, peptides, polypeptides, sugars, ions, and toxins. Further, Higgins provides example of each class of transporter and assays that one of skill in the art could use to assay an ABC transporter family member for the ability to transport a given substrate. Accordingly, one of ordinary skill in the art would have known the broad range of substrates transported by the ABC family of transporters and, therefore, would have been able to test members of these classes of substrates for the ability to inhibit MRP- β .

In view of the foregoing, Applicants respectfully submit that an ordinarily skilled artisan would have been fully equipped with the knowledge provided by virtue of Applicants' disclosure, in combination with the knowledge available to the ordinary skilled artisan at the time, to practice the claimed invention using only routine experimentation. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw this 112, first paragraph rejection.

Objection to the Specification

The Examiner has objected to the amendment filed May 13, 2002, because, "it introduces new matter into the disclosure." Applicants respectfully submit that the amendment to the specification submitted on May 13, 2002 is fully supported by Figure 2

as originally filed. The material added to the paragraph at page 35 is presented in the header of Figure 2 as filed, and therefore, is not new matter.

The Examiner has also objected to the specification because, “it contains an embedded hyperlink and/or other form of browser-executable code.” Applicants have amended the paragraph beginning at page 29, line 16 to remove the hyperlink, thereby rendering this rejection moot.

Rejection of Claims 107-108, 115, 117-118, 120-124, and 129-133

Under 35 USC 112, First Paragraph

The Examiner has rejected claims 107-108, 115, 117-118, 120-124, and 129-133 Under 35 USC 112, first paragraph, as “failing to comply with the written description requirement.” Specifically, the Examiner believes that, “[t]he newly added terminology ‘as determined by the ALIGN algorithm....Gap penalty=4’ in claims 107 and 108 is new matter. This new limitation was not supported by the specification as originally filed.”

As indicated above, the material rejected to by the Examiner as new matter is fully supported by Figure 2 as filed. However, in the interest of expediting prosecution, Applicants have amended claims 107 and 108 to remove the objected to terminology. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw this rejection.

Rejection of Claims 108, 126, and 132 Under 35 USC 112, Second Paragraph

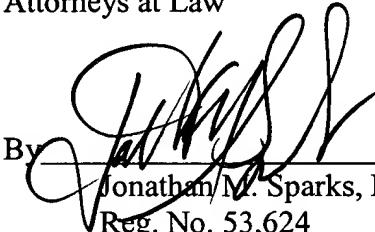
The Examiner has rejected claims 108, 126, and 132 under 35 USC 112, second paragraph, as “being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Applicants have amended claims 108, 126, and 132 to correct the minor informalities in the claims, thereby rendering this rejection moot.

SUMMARY

Entry into the record of the application of the foregoing claim amendments and remarks, and allowance of this application with all pending claims are respectfully requested. If a telephone conversation with Applicants' Attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call (617) 227-7400.

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ABC TRANSPORTERS: From Microorganisms to Man

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INTRODUCTION

All cells and subcellular compartments are separated from the external milieu by lipid membranes. Cell survival requires the regulated and selective passage

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of specific molecules across these membranes, not only to acquire nutrients and excrete waste products, but also for a multitude of regulatory and other functions. The importance of membrane transport to the cell is exemplified by the fact that almost 20% of the *E. coli* genes so far identified are associated with transport functions (Bachmann 1990). Transmembrane transport is mediated by specific proteins associated with the membrane. The characterization of these proteins and their roles in cellular physiology have been a focus of intensive research for many years. It is now apparent that these myriad membrane transport systems can be grouped into a limited number of families. Within any one family, members are related to each other in sequence and molecular mechanisms and probably have a common evolutionary origin. This review considers the largest and most diverse of these families, the ABC superfamily. ABC transporters have received considerable attention recently because they are associated with many important biological processes in both prokaryotes and eukaryotes, as well as with clinical problems such as cystic fibrosis, antigen presentation, and multidrug resistance of cancers.

The designation ABC transporters recognizes a highly conserved ATP-binding cassette, which is the most characteristic feature of this superfamily (Higgins et al 1986; Hyde et al 1990). These transporters have also been referred to as traffic ATPases (Mimura et al 1990). The bacterial uptake systems, the first ABC transporters to be extensively characterized, are frequently termed periplasmic or binding protein-dependent transport systems in recognition of a periplasmic receptor that distinguishes them from other bacterial transporters (Ames 1986). This designation is, however, inappropriate for the superfamily as a whole because many ABC transporters do not require an associated periplasmic component (see below). The term P-glycoprotein or multidrug-resistance family, sometimes used in a general context, is also potentially misleading. The P-glycoproteins are a defined multigene family (Endicott et al 1991; see below): most ABC transporters have no role in multidrug resistance and are not glycoproteins.

Over 50 ABC transporters are now known (Table 1). The majority are from prokaryotic species but, increasingly, eukaryotic examples are being reported. Typically, ABC transporters utilize the energy of ATP hydrolysis to pump substrate across the membrane against a concentration gradient (possible exceptions are discussed below). Each ABC transporter is relatively specific for a given substrate. Nevertheless, the variety of substrates handled by different transporters is enormous: ABC transporters specific for amino acids, sugars, inorganic ions, polysaccharides, peptides, and even proteins have been characterized (Table 1). Some ABC transporters are uptake (import) systems that accumulate substrate within the cell, while others export substrate from the cell: none has yet been identified that can pump in both directions.

Table 1 ABC proteins

Species	System	Substrate	Import/export	Reference
Bacteria				
<i>Salmonella typhimurium</i>	OppABCDF	Oligopeptides	Import	Hiles et al 1987
<i>Streptococcus pneumoniae</i>	AmiABCDEF	Oligopeptides	Import	Alloing et al 1990
<i>Bacillus subtilis</i>	Opp (SpoOK)	Oligopeptides	Import	Perego et al 1991
<i>E. coli</i>	Dpp	Dipeptides	Import	Rudner et al 1991
<i>Bacillus subtilis</i>	DctA	Dipeptides	Import	Abouhamad et al 1991
<i>S. typhimurium</i>	HisJQMP	Hisidine	Import	M. Manson, unpublished
<i>E. coli</i>	HisJQMP	Hisidine	Import	Mathiopoulos et al 1991
<i>E. coli</i>	MalFGK	Maltose	Import	Higgins et al 1982
<i>S. typhimurium</i>	MalFGK	Maltose	Import	Kraft & Leinwand 1987
<i>E. coli</i>				Gilson et al 1982
<i>E. coli</i>				Frosthauer & Beckwith 1984
<i>S. typhimurium</i>	MalFGK	Maltose	Import	Dassa & Hofnung 1985
<i>Enterobacter aerogenes</i>	MalFGK	Maltose	Import	Dahl et al 1991
<i>E. coli</i>	UgpABC	sn-Glycero-3-phosphate	Import	Dahl et al 1991
<i>E. coli</i>	AraFGH	Arabinose	Import	Overduin et al 1988
<i>E. coli</i>	RbsACD	Ribose	Import	Scripture et al 1987
<i>E. coli</i>	GlnHPQ	Glutamine	Import	Bell et al 1986
<i>S. typhimurium</i>	ProU(VWX)	Glycine-betaine	Import	Nohno et al 1986
<i>E. coli</i>	ProU(VWX)	Glycine-betaine	Import	Stirling et al 1989
<i>E. coli</i>	LiVHMGF(UK)	Leucine-isoleucine-valine	Import	Overdier et al 1989
<i>E. coli</i>	PtABC	Phosphate	Import	Gowrishankar 1989
<i>Pseudomonas stutzeri</i>	NooYF	Copper	Import	May et al 1989
<i>E. coli</i>	ChiJD	Molybdenum	Import	Adams et al 1990
<i>E. coli</i>	CysPTWAM	Sulphate-Thiosulphate	Import	Surin et al 1985

Table 1 ABC proteins (Continued)

Species	System	Substrate	Import/export	Reference
<i>E. coli</i>	BtuCDE	Vitamin B12	Import	Friedrich et al 1986
<i>E. coli</i>	FhuBCD	Fe ³⁺ -ferrichrome	Import	Coultou et al 1987
<i>E. coli</i>	FecBCDE	Fe ³⁺ -dicitrate	Import	Kostler & Braun 1986
<i>S. marcescens</i>	StuABC	Fe ³⁺	Import	Staudemrauer et al 1989
<i>Mycoplasma</i>	p37,29,69	?	?	Angerer et al 1990
<i>E. coli</i>	Phn/Psi	Alkyl-phosphonates (?)	Import?	Dudler et al 1988
<i>S. enteritidis</i>	DtrAB	Daunomycin/Doxorubicin	Export	Chen et al 1990
<i>S. enteritidis</i>	TirC	Tylosin	Export	Guilfoile & Hutchinson 1991
<i>S. enteritidis</i>	MsrA	Erythromycin resistance	Export?	Rosteck et al 1991
<i>S. enteritidis</i>	OcclQMP	Oclopine	Import	Ross et al 1990
<i>E. coli</i>	HlyB	Haemolysin	Export	Valdivia et al 1991
Pasturella	LktB	Leukotoxin	Export	Felmlee et al 1985
<i>E. coli</i>	CvaB	Colicin V	Export	Hess et al 1986
<i>Erwinia chrysanthemi</i>	PrtD	Proteases	Export	Strathdee & Lo 1989
<i>Borderella pertussis</i>	CyaB	Cyclolysin	Export	Highlander et al 1989
<i>Streptococcus pneumoniae</i>	ComA	Competence factor	Export?	Gilson et al 1990
<i>Rhizobium meliloti</i>	NdvA	β-1,2-glucan	Export	Letoffe et al 1988
<i>Agrobacterium tumefaciens</i>	ChvA	β-1,2-glucan	Export	Glaser et al 1988
<i>Haemophilus influenzae</i>	BekAB	Capsule polysaccharide	Export	Hui & Morrison 1991
<i>E. coli</i>	KpsMT	Capsule polysaccharide	Export	Stanfield et al 1988
<i>Neisseria</i>	CrtCD	Capsule polysaccharide	Export	Cangelosi et al 1989
<i>E. coli</i>	RsE	Cell division	?	Kroll et al 1988
<i>E. coli</i>	UvrA	DNA repair	None	Husain et al 1986
<i>Rhizobium leguminosarum</i>	NodI	Nodulation	?	Evans & Downie 1986
<i>Rhizobium meliloti</i>	ORF1	?	?	Albright et al 1989

Cyanobacteria				Holland & Wolk 1990
<i>Anabaena</i>	HetA	Differentiation	?	Green et al 1989
<i>Synechococcus</i>	CysA	Sulphate	Import	
Yeast				
<i>S. cerevisiae</i>	STE6	a-mating peptide	Export	Kuchler et al 1989
<i>S. cerevisiae</i>	ADP1	?	?	McGrath & Varsavsky 1989
<i>S. cerevisiae</i>	EF-3	Translation	None	Goffeau et al 1990
Protozoa				Qin et al 1990
<i>Plasmodium</i>	pMDR	Chloroquine	Export	Foore et al 1989
<i>Liesmania</i>	lgpA	Methotrexate/heavy metals	Export	Ouellette et al 1990
Insect				Callahan & Beverley 1991
<i>Drosophila</i>	white-brown	Eye pigments	?	O'Hare et al 1984
<i>Drosophila</i>	Mdr49	Hydrophobic drugs?	?	Dreesen et al 1988
	Mdr65	?	?	Wu et al 1991
			?	Wu et al 1991
Plants				
Liverwort chloroplast	MopX	?	?	Ohyama et al 1986
Animals				
Man	CFTR	Chloride	Channel	Riordan et al 1989
Mouse	CFTR	Chloride	Channel	Tata et al 1991
Xenopus	CFTR	Chloride	Channel	Yonifiji et al 1991
Cow	CFTR	Chloride	Channel	Tucker et al 1992
Dogfish	CFTR	Chloride	Channel	Diamond et al 1991
Man	MDR1	Hydrophobic drugs	Channel	Marshall et al 1991
	MDR3	?	Export	Chen et al 1986
			?	van der Blieck et al 1987

Table 1 ABC proteins (*Continued*)

Species	System	Substrate	Import/export	Reference
Mouse	MDR1	Hydrophobic drugs	Export	Gros et al 1986
	MDR2	?	?	Gros et al 1988
	MDR3	Hydrophobic drugs	Export	Devault & Gros 1990
	MDR3	Hydrophobic drugs	Export	Hsu et al 1989
	Pgp1	Hydrophobic drugs	Export	Endicott et al 1991
	Pgp2	Hydrophobic drugs	Export	Endicott et al 1991
	Pgp3	?	?	Endicott et al 1991
	PM/P70	Polypeptides?	Import into peroxisome (Zellweger syndrome)	Kamijo et al 1990
	RING4-11	Peptides	ER	Gartner et al 1992
	PSF1-PSF2	Peptides	ER	Trowsdale et al 1990
Mouse	HAM1-HAM2	Peptides	ER	Powis et al 1992
	Mpl	Peptides	ER	Spies et al 1990
Rat				Bahram et al 1991
				Monaco et al 1990
				Cho et al 1991
				Deverson et al 1990

A listing of known ABC proteins is presented. While most have been identified on the basis of transport functions, others are only known from gross phenotypes of mutants and may or may not function as transporters (e.g., *FtsE* mutants are defective in cell division). Finally, a few have been identified purely by sequence conservation, and their cellular functions are entirely unknown. Where homologous systems have been characterized in several species, these species are indented and grouped in the listing. For example, the *ami locus* of *Streptococcus* and the *SpdK* locus of *Bacillus* are considered to be functional and evolutionary equivalents of the *Salmonella* Opp system. In some cases (e.g. the putative peptide transporter of the MHC), different names have been accorded the same system even within one species (RING4-11 and PSF1-2).

DOMAIN ORGANIZATION: The Typical ABC Transporter

ABC transporters require the function of multiple polypeptides/protein domains, organized in a characteristic fashion (Figure 1). The typical transporter consists of four membrane-associated domains. Two of these domains are highly hydrophobic and each consists (normally) of six membrane-spanning segments. These domains form the pathway through which substrate crosses the membrane and, in large part, are believed to determine the substrate specificity of the transporter. The other two domains are peripherally located at the cytoplasmic face of the membrane, bind ATP, and couple ATP hydrolysis to the transport process. The sequences of these ATP-binding domains have been highly conserved throughout evolution.

The individual domains of an ABC transporter are frequently expressed as separate polypeptides, particularly in prokaryotic species (e.g. the oligopeptide permease of *S. typhimurium*; Figure 2A). However, there are many examples in which the domains are fused into larger, multifunctional polypeptides. Almost every conceivable type of fusion has now been reported. For example, the two ATP-binding domains of the *E. coli* ribose transporter are fused into a double-sized protein (RbsA; Figure 2B). The two membrane-spanning domains of the iron-hydroxamate transporter of *E. coli* are fused into a single

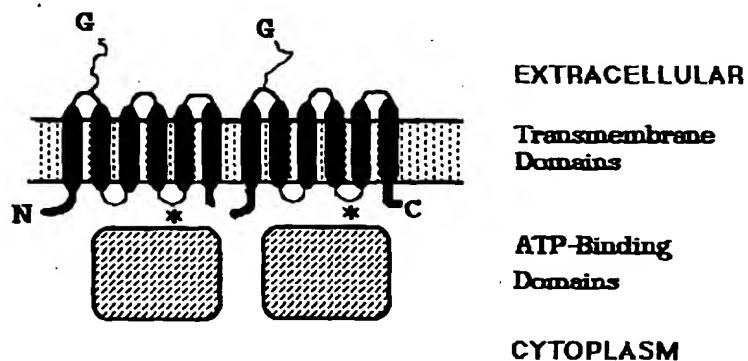


Figure 1 Structural organization of a typical ABC transporter. The core domains of a typical ABC transporter are diagrammed as separate polypeptides (although they are sometimes fused together; see text). The two transmembrane domains each span the membrane six times: a total of twelve transmembrane segments per transporter. The short sequence motif conserved between many of these transporters (Dassa & Hofnung 1985) is indicated by * on the cytoplasmic loops between transmembrane segments 4 and 5 of each domain. This may be involved in interacting with the ATP-binding domains. Potential glycosylation sites of eukaryotic ABC transporters (G) are on the extracellular face of the membrane. The ATP-binding domains are peripherally located at the cytoplasmic face of the membrane, although a segment might extend partway into the membrane through a pore formed by the transmembrane domains. See text for further details.

polypeptide (FhuB; Figure 2C). The putative peptide transporter of the MHC consists of two polypeptides, RING 4 and RING 11, each of which comprises a hydrophobic domain at the N-terminus and an ATP-binding domain at the C-terminus (Figure 2D). Similarly, the *Drosophila* white and brown gene products each consist of a hydrophobic domain fused to an ATP-binding domain: in these proteins, however, the ATP-binding domain is at the N-terminus and the hydrophobic domain is at the C-terminus (O'Hare et al 1984; Dreesen et al 1988). Finally, many eukaryotic ABC transporters, such as the human multidrug resistance P-glycoprotein (Figure 2E) and the cystic fibrosis gene product (Figure 2F), have all four domains fused into a single polypeptide. The natural fusion and separation of domains can also be

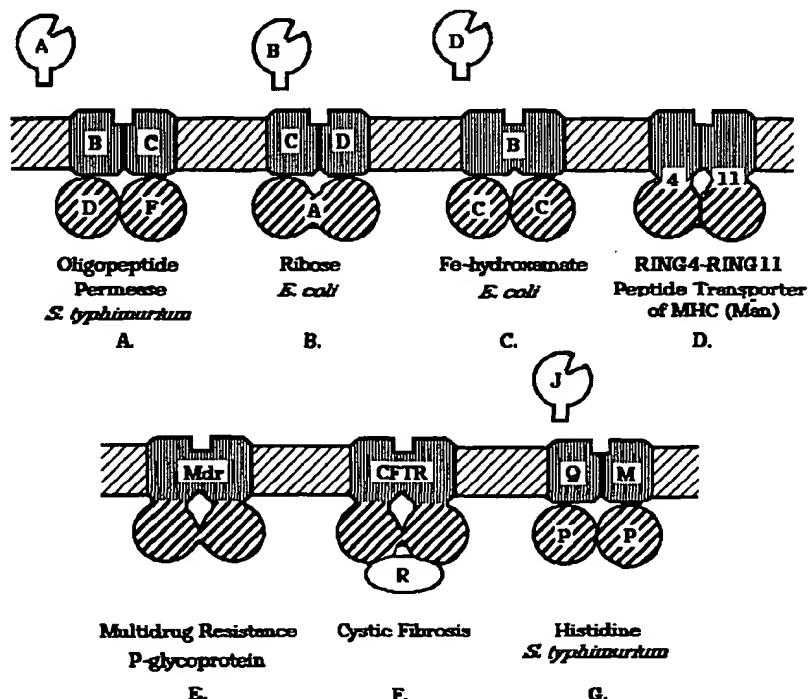


Figure 2 Domain organization of ABC transporters. A typical ABC transporter consists of four domains, two highly hydrophobic membrane-spanning domains (*shaded*), which form the translocation pathway, and two peripheral membrane domains (*shaded*), which couple ATP hydrolysis to the transport process. Certain transporters have additional domains (*unshaded*) that are not part of the core transmembrane translocation mechanism. The domains are often encoded as separate polypeptides; however, they may also be fused together in one of several alternative combinations. References to the original description of these systems are given in Table 1. See text for further details.

mimicked experimentally. Thus the two ATP-binding domains of the *S. typhimurium* peptide transporter (OppD and OppF) can be fused together to give a single functional polypeptide (I. O'Beirne & C. F. Higgins, unpublished data). Similarly, the yeast STE6 α-factor peptide export system, normally a single polypeptide of four domains, can be expressed as two halves yet retain function (Berkower & Michaelis 1991).

The four core domains appear to provide sufficient machinery to mediate the transmembrane translocation of solute. Nevertheless, certain ABC transporters have additional domains that serve regulatory or other peripheral functions. For example, the cystic fibrosis gene product, CFTR, has a fifth domain, the R-domain (Figure 2F), which has no equivalent in any other ABC transporter and serves a regulatory function (Cheng et al 1991; Rich et al 1991; see below). The ATP-binding domain of the maltose transporter (MalK) has a C-terminal extension that has an enzymic function apparently independent of the transport process (Reidl et al 1989). Importantly, all bacterial ABC transporters that mediate solute uptake require a substrate-binding protein located outside the cytoplasmic membrane in the periplasmic space (Figures 2A, C, G). These periplasmic components are essential for the function of the transporter with which they are associated, although they are not integral to the process of transmembrane solute translocation itself (see below). Finally, several bacterial transporters have accessory protein components expressed together with the core domains of the transporter. For example, the HlyB protein, which exports hemolysin across the cytoplasmic (inner) membrane, is coexpressed with the HlyD protein, which participates in hemolysin transport across the outer membrane (Felmlee et al 1985). Similarly, the *lamB* gene, which is cotranscribed with the components of the *E. coli* maltose-maltodextrin transporter, is not required for active transport across the cytoplasmic (inner) membrane, but its product facilitates entry of maltodextrins across the outer membrane (Hengge & Boos 1983).

At first sight, a few ABC transporters appear to lack a full complement of domains. Thus the operon encoding the histidine transporter of *S. typhimurium* includes only a single gene encoding an ATP-binding component, HisP (Higgins et al 1982). However, the transport complex has recently been shown to include HisP in a 2:1 ratio with the other domains, it presumably functions as a homodimer (Kerppola et al 1991; Figure 2G). Indeed, no ABC transporter has yet been shown to function with fewer than the four core domains; in the absence of evidence to the contrary, it is not unreasonable to assume that the four core domains form the basic unit required to mediate solute translocation. Minimalist transporters that appear to lack domains, such as the HlyB hemolysin exporter, which has only one hydrophobic domain and one ATP-binding domain (Felmlee et al 1985), may function as homodimers, although this has yet to be tested experimentally. Finally, a functional transport

complex is generally assumed to consist of one of each of the four core domains rather than a larger oligomeric assembly. However, this is purely an assumption. The twelve transmembrane segments of a monomeric ABC transporter (see below) are, at least potentially, sufficient to mediate transport as the UhpT transporter (a protein with twelve transmembrane segments) has recently been shown to function as a monomer (e.g. Ambudkar et al 1990). Nevertheless, while there is no compelling reason to believe that ABC transporters function as a large oligomeric complex, an unequivocal answer is likely to require the reconstitution of a purified transporter *in vitro*.

THE TRANSMEMBRANE DOMAINS

The "Two-Times-Six" Helix Paradigm

The two transmembrane domains of ABC transporters are highly hydrophobic. Each is predicted, from its sequence, to consist of multiple α -helical segments that could span the membrane. The majority of transporters are predicted to have six membrane-spanning segments per domain (a total of twelve per transporter), with the N- and C-termini on the cytoplasmic face of the membrane and three extracellular and two intracellular loops (Figure 1). The available experimental data, although limited to a few transporters, are consistent with these predictions. Thus the six predicted transmembrane segments of each domain of the oligopeptide permease of *S. typhimurium* have been identified experimentally, using both biochemical and genetic techniques (Pearce et al 1992). The hydrophobic domains of the ABC protein translocators HlyB and PrtD have also been shown to span the membrane six times (Wang et al 1991; Gentschev & Goebel 1992; Delepealaire & Wanderman 1991); these polypeptides seem likely to function as homodimers (see above) so each transport complex has the standard twelve membrane-spanning segments. For the mammalian multidrug resistance P-glycoprotein, studies with epitope-specific antibodies lend direct support to the predicted topology (Yoshimura et al 1989; Georges et al 1990; Zhang & Ling 1991). The predicted topologies also place the glycosylation sites of P-glycoprotein and CFTR appropriately on the extracellular face of the membrane and the ATP-binding domains at the cytoplasmic face (Figure 1). The only data inconsistent with this general model indicate that two of the predicted membrane-spanning segments of one of the hydrophobic domains of P-glycoprotein (but not the equivalent segments of the other domain) may not span the membrane (Zhang & Ling 1991). However, the similarity between the two halves of P-glycoprotein, and analogy with other ABC transporters, make it unlikely that they are actually oriented differently in the membrane. It remains to be determined whether these data reflect the normal *in vivo* organization or are an artifact of the *in vitro* microsome system.

A few ABC transporters do not obviously conform to the two-times-six transmembrane helix paradigm. For example, the MalF protein of the maltose transporter of *E. coli* is predicted to have eight transmembrane segments, and this has been confirmed experimentally (Froshauer et al 1988). Nevertheless, alignment with the equivalent components of other transporters indicates that MalF consists of the six standard transmembrane segments, but has an N-terminal extension with two additional transmembrane segments (Overduin et al 1988; Figure 3): these two N-terminal transmembrane segments can be deleted without loss of MalF function (Ehrmann et al 1990). Another potential exception is the histidine transporter of *S. typhimurium*, which has two transmembrane domains, HisQ and HisM, each predicted to have five, rather than six, transmembrane segments (Higgins et al 1982). Alignment with other transporters (Figure 3) indicates that the usual N-terminal transmembrane segment of each domain may be absent, which places the N-termini on the exterior of the cell. This orientation has yet to be confirmed experimentally, although it may indicate that ten (two-times-five) transmembrane segments provide the minimal unit required to form the translocation pathway itself; the additional N-terminal transmembrane segment(s) of most transporters may simply facilitate correct folding, packing, and orientation within the membrane.

Although the number and identity of the membrane-spanning segments of several ABC transporters are now reasonably well established, it should be

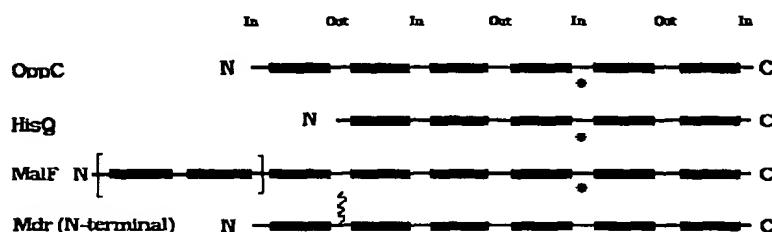


Figure 3 Membrane-spanning segments of ABC transporters. The majority of ABC transporters consist of two related domains, each with six predicted membrane-spanning segments (e.g. OppC, one of the two domains of the oligopeptide transporter of *S. typhimurium*). A short conserved motif is located on the cytoplasmic loop between transmembrane segments 4 and 5 of many bacterial transporters (indicated by *). The N- and C-termini are cytoplasmic. Glycosylation sites on mammalian transporters are in extracellular loops (indicated by a squiggle). Certain transporters do not appear to fit this pattern. Thus, the HisQ and HisM components of the histidine transporter each have only five predicted transmembrane segments. They can be aligned with other transporters and appear to lack transmembrane segment 1. Other proteins, such as MalF of the *E. coli* maltose transporter, have eight membrane spanning segments. The C-terminal six can be aligned with the six transmembrane segments of other transporters: the two N-terminal transmembrane segments can be deleted from the protein without destroying function. See text for further details.

appreciated that there are no experimental data that reflect on the actual structure of the transmembrane segments. They are generally assumed to fold as α -helices, based on computer predictions and the paradigm of bacteriorhodopsin. However, this has not been shown for any transporter. Additionally, it is only possible to guess at how the putative α -helices are packed within the membrane (see below). Resolution of this important question requires structural (NMR, microscopic, or crystallographic) data, which seem unlikely to be obtained in the near future.

Sequence Similarities

Comparison of the amino acid sequences of the transmembrane domains of one transporter with those of another reveals little or no significant similarity (except for a few specific cases: see below). If we take the view that the ABC transporters have a common evolutionary origin (Ames & Higgins 1983; Ames 1986), the low level sequence conservation implies that the structural constraints required for the function of the transmembrane domains can be satisfied by a variety of amino acid combinations. This is not unreasonable if an important function of the transmembrane segments is structural: the requirement to form a hydrophobic α -helical structure may be satisfied by many alternative amino acid sequences. Additionally, the membrane itself will constrain protein folding. Thus one function of the extramembranous loops is to connect one transmembrane segment with the next. Since the position of the transmembrane segments is constrained by their packing within the membrane, the length and primary sequence of the extramembrane loops may be less critical than for soluble proteins, where equivalent loops would play an important role in defining the overall protein fold. Of course, the transmembrane domains are not purely structural elements since they play an important role in determining the substrate specificity of a transporter (see below). Nevertheless, as the specific residues that define the substrate-binding site need not be adjacent in the primary sequence and, as the substrates handled by different ABC transporters are often dissimilar, this is not inconsistent with a lack of sequence conservation.

The only significant sequence conservation between the transmembrane domains of several different ABC transporters is a short motif identified on many bacterial transporters (Dassa & Hofnung 1985), appropriately positioned on a cytoplasmic loop to interact with the ATP-binding domains (Pearce et al 1992; Figures 1, 3). Whether or not it serves this function has yet to be established. Mutation of this motif abolishes transport activity (Dassa 1990). Low level, but significant, sequence similarity can also be detected between the transmembrane domains of the yeast STE6 peptide transporter, the *E. coli* HlyB hemolysin exporter, and human P-glycoprotein (Gerlach et al 1986; Kuchler et al 1989); the functional or evolutionary significance of

this is unclear. Otherwise, the few examples of sequence similarities between transmembrane domains probably reflect relatively recent gene duplication events. For example, the transmembrane domains of rodent P-glycoproteins 1a and 1b (*pgp1* and *pgp2* of hamster; *mdr1* and *mdr3* of mouse) are very closely related; since there is only a single gene in man, these rodent genes are presumably the result of a gene duplication that occurred after mammalian radiation (Endicott et al 1991). Similarly, the proteins of the octopine uptake system of *Agrobacterium* are very closely related to those of the histidine transporter of *S. typhimurium* (Valdivia et al 1991); since the chemical nature of the substrates handled by the two systems is not dissimilar, it seems reasonable to suppose that the octopine system diverged following a relatively recent gene duplication. Finally, there is an example of two systems with closely related transmembrane domains, but rather different substrates: the sn-glycerol-3-phosphate and maltose transporters of *E. coli* (Overduin et al 1988). Sequence similarity does not, therefore, necessarily mean similar substrates.

The two integral membrane domains of an ABC transporter are generally more closely related to each other than they are to the equivalent domains of other transporters in the superfamily. Thus the HisQ and HisM components of the *S. typhimurium* histidine transporter are closely related to each other (Higgins et al 1982), as are the OppB and OppC components of the oligopeptide transporter (Hiles et al 1987). Similarly, the two transmembrane domains of the multidrug resistance P-glycoprotein are more closely related to each other than they are to other transporters (Chen et al 1986b; Gros et al 1986). This similarity implies that the two domains function symmetrically as a pseudodimer. This would be consistent with the view that ABC transporters with a single hydrophobic domain may function as a homodimer (see above).

THE ATP-BINDING DOMAINS

The ATP-binding domains of ABC transporters are their most characteristic feature. Each domain is about 200 amino acids long and the domains from different transporters share considerable sequence identity, varying between 30 and 50% depending on the transporters being compared (Higgins et al 1986; Hyde et al 1990). The sequence identity is generally greater between the two ATP-binding domains of a single transporter than between domains from different transporters: this may reflect functional constraints or may simply be a consequence of concerted evolution. The sequences conserved between the ATP-binding domains include two short motifs associated with many nucleotide-binding proteins (the Walker motifs; Walker et al 1982; Higgins et al 1985, 1986; Figure 4). However, it is important to emphasize

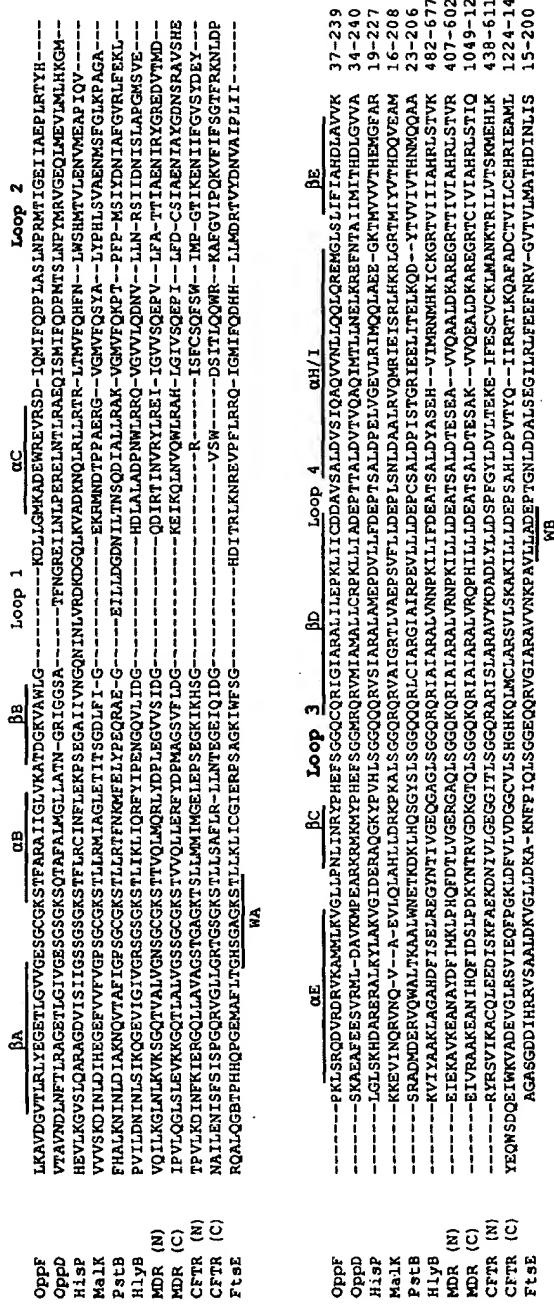


Figure 4 Sequence alignment of ATP-binding domains. A selected number of ATP-binding domains are aligned to illustrate key features. Similar alignments have been published elsewhere (Higgins et al 1986, 1988; Hyde et al 1990; Mimura et al 1991; Riordan et al 1989). The sequence presented illustrates the extent of the domain over which sequence is conserved between transporters. The conserved sequence includes the two Walker motifs characteristic of many nucleotide-binding proteins. These are indicated WA and WB beneath the sequence. Walker motif A corresponds to the P-loop or glycine-rich loop known to be involved in phosphoryl transfer in many nucleotide-binding proteins. Structural motifs (α -helices and β -sheets) are as in the structural modeling of Hyde et al (1990). These are predicted to form the core nucleotide-binding fold. Extending from this core fold are several loops (1 to 4) which extend from the core structure. The loop 2/3 region is of particular interest as it may play a role in coupling the energy of ATP hydrolysis, via conformational changes, to the transport process by directly interacting with the other domains. These loops are also the site of many cystic fibrosis mutations. References to the published sequences are in Table 1. See text for further discussion.

that the sequence identity extends over the entire domain and is far more extensive than the two short Walker motifs. This clearly distinguishes the ABC domains from other nucleotide-binding proteins: not all proteins with the Walker motifs are ABC proteins.

A sequence alignment of the ATP-binding cassettes is presented in Figure 4 and illustrates certain key features. The domains can be defined by the extent of the conserved sequence (Higgins et al 1988; Hyde et al 1990) and by the smallest polypeptides that include these conserved sequences: the FtsE and PstB proteins seem to define the minimal domain (Higgins et al 1988). Some domains, of course, form part of larger, multifunctional polypeptides (see above). The vast majority of ABC domains are associated with membrane transport events (Higgins et al 1986, 1988; Table 1). Nevertheless, a few apparently typical ABC proteins serve alternative functions. Most obvious of these is the UvrA protein, which is a cytoplasmic enzyme involved in DNA repair. This multidomain polypeptide includes two typical ABC domains interrupted by DNA-binding zinc fingers (Doolittle et al 1986); the UvrA protein binds and hydrolyzes ATP as part of its repair function (Seeberg & Steinum 1982; Mazur & Grossman 1991). Elongation factor EF-3 of yeast also includes an ABC domain, yet is clearly not a transport protein; the role of ATP in its function is still unclear (Qin et al 1990). Other ABC proteins may or may not be transport-associated. For example, the biochemical function of the FtsE protein, required for *E. coli* cell division (Gill et al 1986), is unknown. In conclusion, most ABC domains are transport-associated; however, some appear to have been sequestered during evolution to serve alternate functions. Thus identification of an ABC domain by sequence alignment alone does not necessarily imply a role in transport. Nevertheless, it is reasonable to expect that ABC domains function by similar mechanisms to couple ATP hydrolysis to an appropriate biological event.

The ATP-binding domains are highly hydrophilic, include no potential membrane-spanning segments, and would not normally be expected to span the membrane. Indeed, the available data are consistent with a peripheral location, tightly associated with the cytoplasmic face of the membrane (as depicted in Figure 1). The use of epitope-specific antibodies showed that the ATP-binding domains of P-glycoprotein are only accessible from the cytoplasmic side of the membrane (Georges et al 1990; Yoshimura et al 1989). Similarly, the ATP-binding components of the bacterial oligopeptide and histidine transporters, OppF and HisP respectively, are less accessible to proteolysis from the exterior of the cell than from the cytoplasm (Gallagher et al 1989; Kerppola et al 1991). The observation that the MalK component of the maltose transporter is cytoplasmic in the absence of the membrane-associated components also points to a peripheral association with the cytoplasmic face of the membrane (Shuman & Silhavy 1981). Finally, this location

is entirely consistent with a role in coupling ATP hydrolysis to transport (see below).

For transporters where the ATP-binding domain is part of a multidomain polypeptide (e.g. P-glycoprotein), the transmembrane domains serve as a membrane anchor. Even when expressed as separate polypeptides (i.e. many bacterial systems; Figure 1), the ATP-binding domains still associate with the membrane in a complex with the transmembrane components (Silhavy & Shuman 1981; Kerppola et al 1991; Davidson & Nikaido 1991). These interactions must be specific as the ATP-binding domain from one transporter cannot normally replace that of another. Indeed, these interactions appear to induce a conformational change in the ATP-binding domain that alters its biochemical properties (Reyes & Shuman 1988; Davidson & Nikaido 1990). The residues involved in these interactions are unknown, although gene-fusion studies show that specificity cannot reside in the N-terminus (Schneider & Walter 1991). Residues that are exposed as loops extending from the core ATP-binding structure (the loop 2/3 region; Figure 4) may be involved. Models have been presented in which the ATP-binding domains span the membrane (Ames & Higgins 1983; Ames 1985, 1986). These were based primarily on genetic suppression data obtained for the histidine transporter, which showed that mutations altering the periplasmic component (*hisJ*) can be suppressed by mutations in the gene encoding the ATP-binding domain (HisP) (Ames & Spudich 1976). The simplest interpretation of these data is that the HisJ and HisP proteins interact and consequently HisP must be exposed at the periplasmic face of the membrane. However, recent data provide an alternative explanation. The mutant HisP protein facilitates transport and ATP hydrolysis even in the total absence of the periplasmic HisJ protein (Petronilli & Ames 1991); there is, therefore, no necessity to propose a direct interaction or that HisP is exposed at the external face of the membrane.

There is no doubt that the ATP-binding domains are peripheral membrane components. Nevertheless, the possibility that a segment of the ATP-binding domain protrudes into or through a pore generated by the transmembrane domains cannot be excluded (Kerppola et al 1991). Structural models of the ATP-binding domains predict sequences extending from the core ATP-binding structure, which could potentially fulfill such a role (the loop 2/3 region; Figure 4; see below). It has also been suggested that HisP (the ATP-binding domain of the histidine transporter) may be accessible to proteases from outside the cell when the transmembrane components of the transporter are present (Kerppola et al 1991). This point remains to be resolved.

Each ABC transporter has two ATP-binding domains and both are required for function. Thus elimination of either one of the two domains of the Opp oligopeptide transporter abolishes function (Hiles et al 1987). Introduction of a mutation into either one of the two ATP-binding domains of P-

glycoprotein (Azzaria et al 1989), or STE6 (Berkower & Michaelis 1991), dramatically reduces transport activity. It is not clear, however, whether the two ATP-binding domains are functionally equivalent. For transporters such as the histidine permease of *S. typhimurium* the two ATP-binding subunits are identical (Kerppola et al 1991), which suggests equivalency. The estimated stoichiometry of two ATP molecules hydrolyzed for each molecule of substrate transported (Mimmack et al 1989) is also consistent with two equivalent domains, each hydrolyzing one ATP molecule per transport cycle. The only experimental data suggesting a functional asymmetry are for CFTR. Many CF mutations fall in the first nucleotide-binding domain (Cutting et al 1990; Kerem et al 1990), and a mutation in the C-terminal nucleotide-binding domain that might be expected to disrupt ATP hydrolysis does not appear to inhibit CFTR function (Anderson et al 1991c). In conclusion, the specific role of each nucleotide-binding domain remains to be clarified.

In order to understand the molecular mechanisms by which the ATP-binding domains couple ATP hydrolysis to the transport process, structural data are required. In the absence of an experimentally determined structure, the domains have been modeled based on the known structures of adenylate kinase and *ras* p21 (Hyde et al 1990; Mimura et al 1991). The fact that these two independent modeling exercises generated similar structures by entirely different procedures is encouraging. The core of both models is a nucleotide-binding fold, which includes the five hydrophobic β -sheets of the Rossman fold and the glycine-rich P-loop (Walker motif A), appropriately positioned to interact with ATP and mediate phosphoryl transfer (for the sequences corresponding to the various structural motifs, see Figure 4). Extending from the core nucleotide-binding fold are loops that have no direct counterpart in adenylate kinase. In one model these sequences are folded as two separate loops (designated loops 2 and 3; Hyde et al 1990); in the other model they are folded as one large loop (Mimura et al 1991). This difference is a consequence of a slightly different sequence alignment and is unlikely to be clarified until an experimentally determined structure is available. However, the important feature is not whether they fold as one loop or two, but that both models predict these same sequences protrude from the core nucleotide-binding fold.

The available data are entirely consistent with these model structures. Thus mutation of residues predicted to form part of the core ATP-binding fold of the yeast STE6 protein (Berkower & Michaelis 1991), the histidine transporter (Shyamala et al 1991), and the multidrug resistance P-glycoprotein (Azzaria et al 1989) drastically reduce function. Labeling studies with 8-azido-ATP are also consistent with the proposed nucleotide-binding fold (Mimura et al 1990). The models also predict that sequences forming the loops extend from the core structure will not be directly involved in binding ATP. This is supported by the observations that mutations in the loop regions of the histidine

transporter generally have little effect on ATP binding (Petronilli & Ames 1991; Shyamala et al 1991). Equivalent mutations in the STE6 protein (Berkower & Michaelis 1991) and the cystic fibrosis protein (Drumm et al 1991) do not abolish function, and insertions can be introduced into the loop 2/3 region without loss of activity (Doolittle et al 1986; Schneider & Walter 1991). Furthermore, the finding that temperature-sensitive mutations in the *ftsE* gene (encoding an *E. coli* ABC protein essential for cell division) all alter the predicted loops (Gibbs et al 1992) supports the view that they are flexible and located outside the core nucleotide-binding fold. A working model consistent with the available data predicts that the ABC proteins have a tightly folded core structure that binds and hydrolyzes ATP; loops that extend from this core structure interact with other components of the transporter and couple, presumably via a conformational change, the energy of ATP hydrolysis to transport or whatever cellular process the protein is associated with. Possible mechanisms are discussed more fully below.

PERIPLASMIC-BINDING PROTEINS

The first ABC transporters to be characterized were the binding protein-dependent transporters of Gram-negative bacteria. One feature that distinguished them from other active transport systems was their sensitivity to cold osmotic shock (Berger 1973; Berger & Heppel 1974), which resulted in the loss of specific substrate-binding proteins from the periplasm (the compartment between the inner and outer membranes of Gram-negative bacteria). Subsequent genetic studies demonstrated that these periplasmic proteins are absolutely required for the function of the transport system with which they are associated. They are relatively easy to purify and many have been studied in considerable detail. They vary in size from 25 kd (histidine; Higgins & Ames 1981) to 59 kd (oligopeptide; Hiles & Higgins 1986), and there is little sequence conservation between binding proteins for different substrates. The only exceptions are the pairs of periplasmic proteins that interact with the same core transmembrane complex (e.g. the histidine and lysine-arginine-or-nitro-arginine-binding proteins, which deliver substrates to the HisMQP complex in the membrane; Higgins & Ames 1981). Several binding proteins have been crystallized and their three-dimensional structures determined (reviewed in Adams & Oxender 1989; Quiocho 1990; Vyas et al 1991). All have a similar structure with two globular domains and a cleft between that forms the substrate-binding site. Whatever the nature of the substrate (i.e. charged, hydrophobic, etc) it appears to be bound via hydrogen bonds (Quiocho 1986; Pflugrath & Quiocho 1988).

The periplasmic proteins serve as the initial receptor for transport, delivering

substrate to the membrane-bound components. Importantly, the *in vitro* binding specificities and affinities measured for the purified proteins correspond well with *in vivo* characteristics of the transport process, which implies that binding provides the rate-limiting step for transport (Miller et al 1983). The proteins undergo a conformational change upon binding substrate that traps substrate in the cleft between the two domains (The Venus flytrap model; Sack et al 1989; Mao et al 1982). This conformational change enables the binding protein to interact with the appropriate complex of membrane proteins; this interaction does not seem to occur in the absence of bound substrate (Prossnitz et al 1988). Interaction of the binding protein-substrate complex with the membrane-associated transport components has been demonstrated both genetically and biochemically and appears to involve both domains of the periplasmic protein and both hydrophobic transmembrane domains (Treptow & Shuman 1985, 1988; Kossman et al 1988; Prossnitz et al 1988).

Although the periplasmic components are required for the function of the transporter with which they are associated, they are not integral to the mechanism of transmembrane translocation itself. Many ABC transporters, including all known eukaryotic systems, do not require an equivalent component (Figure 1). Additionally, mutants of bacterial uptake systems can be isolated that function in the absence of the periplasmic component (Shuman 1982; Speiser & Ames 1991). Thus the periplasmic proteins are probably best considered as accessory components, a specific adaptation to particular circumstances. What are these circumstances? Two of the most common suggestions, that the binding proteins increase the effective concentration of substrate in the periplasm, or that they enhance the affinity of otherwise binding protein-independent transport systems, can been persuasively excluded (Hengge & Boos 1983). It has been suggested that binding proteins facilitate the movement of substrate within the periplasm: the periplasm of Gram-negative bacteria is a gel-like matrix and, at low substrate concentrations, diffusion may be limiting (Brass et al 1986). However, the recent identification of binding proteins in Mycoplasma and other Gram-positive species, which do not have a periplasm (Gilson et al 1988; Perego et al 1991; Dudler et al 1988), argues against this. An adaptation of this hypothesis is that the binding proteins enhance transport by restricting diffusion to two, rather than three, dimensions. It is not unreasonable that diffusion of the binding protein-substrate complex in two dimensions enhances the efficiency at which substrate is delivered to the membrane transport complex, compared with three-dimensional diffusion of the unbound substrate in solution. In Gram-positive species the binding proteins are anchored to the membrane by a lipid group, and diffusion is consequently restricted to two dimensions. In Gram-negative species the dimensions of the periplasm also effectively restrict diffusion of

the binding protein to two dimensions. Indeed, in Gram-negative bacteria the periplasmic proteins still function efficiently when anchored to the membrane via a noncleavable signal sequence (Fikes & Bassford 1987).

A final possibility is that the periplasmic protein imposes directionality on transport. No ABC transporter has yet been found that can mediate both uptake and export of a substrate, and it does not seem to be possible to convert an importer into an exporter by a few mutational changes (C. F. Higgins, unpublished data). Furthermore, comparison of the membrane-associated proteins of uptake and export systems does not identify any feature that allows the two to be distinguished. Yet all known uptake systems require a periplasmic component, while no export systems does. Given the number of transporters that have now been characterized (Table 1), this may be more than a coincidence. Since interaction of the binding-protein-substrate complex with the membrane-associated domains is required for ATP hydrolysis (Petronilli & Ames 1991), an induced conformational change in the membrane-associated domains of the transporter is implied, consistent with such a model. Nevertheless, data have yet to be presented that directly support or refute this model.

SUBSTRATE SPECIFICITY

ABC transporters have been identified for almost every class of substrate imaginable, including sugars, peptides, inorganic ions, amino acids, oligopeptides, polysaccharides, and proteins (Table 1). Not only are these substrates chemically very different, but they also vary enormously in size. The mechanism by which such diversity is achieved, while each transporter retains a high degree of selectivity for its own particular substrate, presents an intriguing problem.

For bacterial uptake systems, the periplasmic-binding proteins play a role in determining substrate specificity (see above). However, as binding protein-independent mutants still exhibit substrate selectivity (Treptow & Shuman 1985; Petronilli & Ames 1991), the four membrane-associated components must also play a role. For systems that do not have a binding protein, the membrane-associated domains must be involved. The weight of evidence points to the transmembrane domains, rather than to the ATP-binding domains, as the primary determinants of substrate specificity. Thus mutations that change the selectivity of a transporter invariably alter the transmembrane domains. For example, mutations that alter the selectivity of the histidine transporter of *S. typhimurium* from L-histidine to L-histidinol delete four amino acids from a membrane-spanning segment of the transmembrane domain HisM (Payne et al 1985). Mutations that allow the maltose transporter of *E. coli* to transport the analogue *p*-nitrophenyl- α -maltoside (not normally a substrate)

also alter the transmembrane domains (Reyes et al 1986). A mutation within transmembrane segment 11 (TM11) of P-glycoprotein modulates substrate specificity (Gros et al 1991), and changes in TM11 of pfMDR from *Plasmodium* are associated with chloroquine resistance and may alter substrate recognition (Foote et al 1990). Changing charged residues in the transmembrane segments of CFTR, by site-directed mutagenesis, can alter its ion selectivity (Anderson et al 1990b). Besides the membrane-spanning segments themselves, extramembrane loops connecting these segments can also influence substrate specificity. Thus a mutation in the first cytoplasmic loop of the multidrug resistance P-glycoprotein, between transmembrane helices 2 and 3 (G185V), increases resistance to colchicine compared with vinblastine (Choi et al 1988), although this mutation may alter a step other than initial substrate recognition (Safa et al 1990). Sequences in the cytoplasmic loops following transmembrane helices 7 and 12 of the yeast STE6 α -factor transporter resemble the α -factor receptor STE3 and have been suggested as possible sites for substrate recognition (Kuchler et al 1989). Finally, complementing these genetic data, biochemical evidence that the transmembrane domains of P-glycoprotein can be specifically labeled by various substrates and substrate analogues shows that they are involved in substrate binding (Bruggemann et al 1989; Yoshimura et al 1989; Morris et al 1991; Greenberger et al 1991).

There is no evidence that the ATP-binding domains of ATP transporters contribute directly to substrate specificity, although the suggestion that part of this domain (the loop 2/3 region; see above) might protrude into a pore formed by the transmembrane domains is compatible with such a role (Kerppola et al 1991). Interestingly, sequences in the loop 2/3 region of the ATP-binding domain of the vitamin B12 transporter, BtuD, are related to the vitamin B12-binding site on the outer membrane receptor (Friedrich et al 1986); the significance of this is unknown. In contrast, experiments with chimeric *mdr* genes argue against a role for the ATP-binding domains in determining substrate specificity (Buschman & Gros 1991). The *mdrl* gene of mouse confers drug resistance while the *mdr2* gene does not: a chimeric molecule in which the ATP-binding domains of *mdrl* are replaced with those of *mdr2* still transport drugs, while replacement of as few as two transmembrane segments of *mdrl* with the equivalent segments from *mdr2* abolishes drug transport.

It is not unreasonable to assume that residues that contribute to substrate recognition reside on several different transmembrane segments and extramembranous loops. Thus until the three-dimensional organization of the transmembrane domains is elucidated, it is unlikely that amino acids involved in determining substrate specificity will be clearly defined. An important consequence is that it is not yet possible to predict substrate specificity of an

ABC transporter, or even the chemical class of substrate, from primary sequence data alone. Only if the transmembrane domains are very closely related to those of a transporter from another species is it reasonable to guess (with caution) that they handle similar substrates. For example, the transmembrane domains of the *ami* and *SpoOK* loci of *Streptococcus* and *Bacillus subtilis*, respectively, were found to share extensive sequence identity with those of the oligopeptide transporter of *S. typhimurium* and were subsequently shown to serve similar functions (Alloing et al 1990; Perego et al 1991). Nevertheless, even close similarities can be misleading: the Mal and Ugp transport systems of *E. coli* are closely related yet handle different substrates (Overduin et al 1988), and the two human *mdr* genes are very similar to each other, yet only one is able to mediate drug transport.

THE ROLE OF ATP: Coupling Energy to Transport

The mechanisms of energy coupling to transport have been most extensively studied for the ABC transporters of Gram-negative bacteria. The energy requirements for binding protein-dependent transporters were distinguished from those of other transport systems nearly twenty years ago, based upon differential sensitivity to metabolic inhibitors (Berger 1973; Berger & Heppel 1974). These seminal studies led to the suggestion that ATP hydrolysis provides the driving force for substrate accumulation, in contrast to other transporters that appeared to respond to transmembrane electrochemical gradients (pmf). Subsequently, however, the role of ATP generated considerable controversy. Alternative energy sources such as acetyl phosphate (Hong et al 1979), NADPH (Gilson et al 1982), and lipoic acid (Richarme 1985) were proposed, and a requirement for the electrochemical gradient was frequently invoked (e.g. Plate 1979; Hunt & Hong 1983; Ames 1986). Nevertheless, data obtained in the past three years have effectively resolved this issue, and there is now little doubt that ATP hydrolysis by the ABC transporters themselves provides the driving force for solute accumulation (reviewed by Higgins 1990; Ames & Joshi 1991).

The first direct evidence of a role for ATP was the recognition of a consensus ATP-binding motif (the Walker motif) on subunits of several bacterial transporters (Higgins et al 1985, 1986; Figure 4; see above): these conserved motifs have been found on all ABC proteins that have subsequently been characterized. The bacterial OppD, HisP, and MalK proteins (Higgins et al 1985; Hobson et al 1984), and mouse P-glycoprotein (Azzaria et al 1989) have been shown to bind ATP and/or a variety of ATP-affinity analogues. The K_m of the histidine transporter for ATP is estimated to be about 100 μM , appropriately less than the normal intracellular ATP concentration (Ames et al 1989). The ABC proteins not only bind ATP, but ATP-binding is essential for function. Thus mutation of the ATP-binding site

of several ABC transporters inhibits activity (Azzaria et al 1989; Berkower & Michaelis 1991; Shyamala et al 1991; Anderson et al 1991c; Gill et al 1992). Additionally, in cell membrane-derived vesicle systems, histidine and maltose transport by *E. coli* and drug transport by human P-glycoprotein show an absolute requirement for ATP (Dean et al 1989; Prossnitz et al 1989; Horio et al 1988).

A requirement for ATP does not necessarily imply that hydrolysis energizes transport. ATP binding could, potentially, serve a structural or regulatory role. However, non-hydrolyzable analogues of ATP are unable to support active transport (Horio et al 1988; Ames et al 1989), and recent studies have demonstrated unambiguously that ATP hydrolysis occurs concomitant with transport. In one approach the histidine and maltose transporters of *S. typhimurium* and *E. coli*, respectively, were reconstituted in vesicles: transport was found to be totally dependent on ATP hydrolysis, and vice versa (Bishop et al 1989; Dean et al 1989). In an alternative approach, *in vivo* studies on several *E. coli* transporters demonstrated that ATP hydrolysis is dependent upon, and occurs concomitantly with, substrate translocation (Mimmack et al 1989); ATP consumption was not observed for non-ABC transporters, which are linked to the electrochemical gradient. Finally, purified UvrA protein (an ABC protein not associated with transport) and partially-purified P-glycoprotein hydrolyze ATP (Seeberg & Steinum 1982; Hamada & Tsuruo 1988). There is, therefore, no doubt that ATP hydrolysis is required for transport and that domains of the transporters themselves can bind ATP. The simplest and presumably correct interpretation is that the ABC domains themselves hydrolyze ATP and directly couple the energy of hydrolysis to the transport process. Nevertheless, this has not been formally demonstrated, and the possibility that the high energy group of ATP is transferred to another molecule that then interacts with the transporter cannot be excluded (although even in this case, ATP would still provide the driving force for transport). Final resolution of this point will require reconstitution of the purified protein into artificial lipid membranes.

It should be noted that besides ATP, GTP and CTP can also energize histidine transport in vesicles (Bishop et al 1989) and substitute for ATP in CFTR function (Anderson et al 1991c). However, the low cytoplasmic pools of these nucleotides and their poor affinities for the transport proteins (Higgins et al 1985; Hobson et al 1984) make it unlikely that they serve a significant role in energizing transport *in vivo*. Despite earlier suggestions (Ames 1986), it also seems clear that the electrochemical gradient plays no significant role in the normal function of ABC transporters: thermodynamic considerations preclude the high degree of substrate accumulation being supported by the electrochemical gradient (Hengge & Boos 1983); the movement of protons cannot be detected together with substrate (Darawalla et al 1981); and the judicious use of uncouplers in vesicle experiments (Bishop et al 1989)

effectively excludes a role for the electrochemical gradient. The inhibition of ABC transporters sometimes observed following perturbation of the electrochemical gradient may well be a consequence of altered intracellular pH (Driessens et al 1987; Joshi et al 1989). It should be noted, however, that hemolysin export by HlyB requires the pmf (Koronakis et al 1991): protein export by the normal *sec* pathway also requires both ATP and the pmf (reviewed by Geller 1991). Protein translocation presumably requires additional steps to those required for export of a small molecule (see below) since the transmembrane pathway must remain open for a relatively extended period as the protein is fed through: the pmf may be required for these later events.

The stoichiometry of ATP hydrolysis is not firmly established. On thermodynamic and energetic grounds the number of ATP molecules hydrolyzed per molecule of substrate transported must be close to unity: for energy sources such as maltose, a higher stoichiometry would require more energy to take it into the cell than could be generated from it. Indirect estimates of the stoichiometry of maltose uptake based on growth yields give a value close to one (Muir et al 1985). In vesicle systems, stoichiometries of 5 (Bishop et al 1989) and 1.4 to 17 (Davidson & Nikaido 1990) have been reported, although these high and variable stoichiometries may reflect futile cycling of the transporter in vesicle systems. More direct estimates using whole cells suggest a stoichiometry of close to two ATP molecules hydrolyzed per molecule of substrate transported (Mimmack et al 1989), a value consistent with two ATP-binding domains per transporter. Although a stoichiometry of greater than one appears inefficient, it may be the penalty required in order to accumulate substrate against very large concentration gradients. Importantly, whether the stoichiometry is one or two, there is undoubtedly stoichiometric coupling of ATP hydrolysis to the transport process: this conclusion is important when considering channel functions associated with ABC transporters (see below).

How is the energy of ATP hydrolysis coupled to the transport process? In the absence of structural data, our understanding is akin to "hand-waving". There is no evidence that a phosphorylated protein intermediate is involved and, although this is negative evidence, it seems unlikely that such an intermediate would have been missed (Ames & Nikaido 1981; C. F. Higgins, unpublished data). (Note: although several eukaryotic ABC transporters are phosphorylated, these are regulatory events and have nothing to do with the mechanisms of energy coupling; see below.) The mechanisms, therefore, presumably differ from the P-type ATPases, which involve phosphorylated intermediates (Pedersen & Carafoli 1987). The characteristic sequence differences between ABC transporters and the P-type ATPases also suggest that they are mechanistically distinct. In the absence of phosphorylated intermediates it is generally assumed that ATP binding and hydrolysis induces a conformational change in the ATP-binding domain, which is transmitted,

via domain-domain interactions, to the transmembrane subunits that mediate translocation across the membrane. The loop 2/3 region of the ATP-binding domains (Figure 4; see above) is a good candidate for involvement in this conformational transduction. Residues in adenylate kinase and the *ras* p21 proteins, which are equivalent to these loops, are precisely those which undergo conformational changes upon nucleotide-binding and hydrolysis (Schulz et al 1990; Diederichs & Schulz 1990; Jurnak et al 1990). Additionally, mutations in the loop region can uncouple ATP hydrolysis from the transport event, yet do not alter ATP binding or hydrolysis (Petronilli & Ames 1991). Finally, a DNA-binding zinc finger is inserted into the loop region of UvrA (a non-transporting ABC protein; Doolittle et al 1986). Because this protein couples ATP hydrolysis to interactions with DNA, this strongly implies that the loop region is involved in transducing ATP-dependent conformational changes.

COVALENT MODIFICATION

Covalent modification of proteins frequently serves a regulatory role, particularly in eukaryotic cells. Both P-glycoprotein and CFTR are glycosylated, presumably during trafficking through the Golgi. Nevertheless, the carbohydrate can be removed, either biochemically or by mutating the glycosylation site, without dramatically affecting function (Gregory et al 1991; Germann et al 1990). Both P-glycoprotein and CFTR are also phosphorylated, although it must be stressed that this is independent of energy coupling. In the case of CFTR, phosphorylation by protein kinase A is required to activate channel function (Cheng et al 1991). Phosphorylation by protein kinase C has been implicated in regulating of P-glycoprotein activity (e.g. Chambers et al 1990). These and other modifications are clearly of considerable importance in regulating the activity of specific transporters. Nevertheless, they are specific to individual transporters, rather than being related to the general mechanisms by which ABC transporters function, and fall outside the scope of this review.

CELLULAR FUNCTIONS OF ABC TRANSPORTERS

As can be seen from Table 1, ABC transporters can serve a wide variety of cellular roles. A number that illustrate particular physiological or mechanistic points with general implications are discussed below.

Nutrient Uptake

The ability of bacteria to grow and compete depends upon the efficiency with which they can obtain and scavenge nutrients. Even for bacteria such as *E.*

coli, which can grow in a defined medium, growth is much more rapid when nutrients such as amino acids are provided. It is energetically more efficient to take up compounds from the external environment than to synthesize them intracellularly. For example, histidine synthesis requires 41 ATP equivalents (Ames 1986), while only 1–2 ATPs are required to transport histidine into the cell. Therefore, it is not surprising that bacteria have a plethora of nutrient uptake systems, to a first approximation one for each required substrate.

Several families of active transporters have been defined in bacteria besides the ABC transporters. The PTS (phosphotransferase) systems are energized by phosphorylation of the substrate as it crosses the membrane; this necessarily restricts the range of substrates to sugars and their derivatives (Lengeler et al 1990). In contrast, the twelve-membrane-spanning-helix transporters resemble the ABC transporters in that they can handle a wide range of different substrates and are found in both pro- and eukaryotic species (reviewed by Henderson 1991). They differ from ABC transporters in that they are energized by the electrochemical gradient or by co- or countertransport, and any one transporter can translocate solute in either direction depending upon the electrochemical gradient. For some substrates in *E. coli*, both an ABC transporter and a twelve-transmembrane-helix transporter are found. Why this apparent duplication of effort? It is possible that under certain growth conditions changing energy status might favor the use of transporters energized by alternate means. As a general rule, however, the twelve-transmembrane-helix transporters are low affinity but high capacity systems and, for thermodynamic reasons, cannot accumulate substrate against large concentration gradients (Hengge & Boos 1983). They seem best suited for bulk uptake of carbon or nitrogen sources for growth. In contrast, ABC transporters are low capacity but high affinity systems and can accumulate substrate against very large concentration gradients (>10,000-fold) and are most appropriate for a scavenging role. Consistent with this view is the observation that the majority of twelve-transmembrane-helix transporters handle sugars and other potential growth substrates (e.g. lactose, xylose, proline), while nutrients that are generally scarce and required in small amounts (e.g. vitamin B12; iron-chelates) are taken up via an ABC transporter.

For cells that experience few fluctuations in external nutrient availability and do not need to scavenge, a role for ABC transporters in nutrient uptake may be less apparent. This is certainly the case for mammalian cells where the majority of nutrient uptake systems that have been characterized appear to be linked to ion gradients rather than ABC transporters. Additionally, endocytosis in mammalian cells may obviate the need for a great variety of specific uptake systems. Indeed, no ABC transporter involved in uptake has yet been characterized in eukaryotic cells. Nevertheless, as only a limited number of mammalian transporters have been characterized, further developments may bring surprises.

Protein Export

The majority of proteins exported from both pro- and eukaryotic cells are synthesized as precursors with a signal peptide that directs them to their extracellular location. In bacteria, signal peptide-dependent protein secretion is mediated by the Sec protein complex, which provides the principal pathway for export. A small number of exported proteins lack a typical signal peptide and are apparently exported in a Sec-independent fashion. Proteins exported by signal peptide-independent mechanisms frequently serve peripheral functions associated with virulence and are generally encoded together with the machinery required for their export. Signal peptide-independent export is mediated by ABC transporters (Blight & Holland 1990). The paradigm is the 107 kd HlyA hemolysin polypeptide of *E. coli*, which is encoded together with a protein required for its activation (HlyC), a protein required for export across the outer membrane (HlyD), and the ABC protein (HlyB), which mediates hemolysin export across the cytoplasmic membrane (Felmlee et al 1985). Other ABC proteins export proteases from *Erwinia* (Letoffe et al 1990), cyclolysin from *Bordetella pertussis* (Glaser et al 1988), and colicin V from *E. coli* (Gilson et al 1990). Each of these exporters is relatively specific for its polypeptide substrate, although a degree of functional complementation has been observed (Fath et al 1991; Delepelaire & Wandersman 1990; Letoffe et al 1991; Guzzo et al 1991; Masure et al 1991). For most transported proteins such as HlyA, specificity resides in the C-terminal amino acids (Gray et al 1986; Koronakis et al 1989), although for colicin V the recognition sequence is N-terminal (Fath et al 1991). It appears that recognition requires structural motifs rather than specific sequence elements (Stanley et al 1991; Kenny et al 1992).

In eukaryotic cells, several exported proteins also lack a cleavable signal peptide and, therefore, are presumably translocated by a nonconventional mechanism. The only such transporter identified to date is the STE6 protein of yeast (Kuchler et al 1990; McGrath & Varshavsky 1989). STE6 mediates export of the α -factor mating peptide which, unlike the corresponding α -factor, has no signal peptide and is exported in a SEC-independent manner. STE6 is relatively specific for its substrate and will not mediate export of most other polypeptides/proteins. Since various mammalian proteins such as interleukin IL-1 and fibroblast growth factors lack signal sequences, an ABC protein (yet to be identified) may mediate export of these and other polypeptides.

Intracellular Membranes

Most ABC transporters mediate import or export across the cytoplasmic membrane. However, there is no inherent reason why ABC transporters should not also mediate transport across intracellular membranes. An ABC protein that is a major component of the peroxisome membrane (Kamijo et al 1989) may import proteins into this organelle (Gartner et al 1992). pfMDR of

Plasmodium is located on the digestive vacuole membrane (Cowman et al 1991), and it has been suggested that the CFTR protein might function in the membrane of intracellular vesicles (Barasch et al 1991). The best example of an ABC protein with an intracellular function is the putative peptide transporter of the endoplasmic reticulum (ER). Antigen presentation by MHC class I molecules requires that viral polypeptides are degraded in the cytoplasm and the resultant peptides transported into the ER where they complex with the class I molecule before trafficking to the cell surface (Townsend & Bodmer 1989). These small peptides lack a signal peptide and all available data are consistent with the RING4-RING11 complex (Figure 1; for alternative designations see Table 1) mediating peptide transport into endoplasmic reticulum (Kelly et al 1992). Antibody localization also places RING4-RING11 in the endoplasmic reticulum (A. Townsend, personal communication). Although the RING4-RING11 complex probably transports a wide variety of small peptides, it is interesting to consider whether polymorphisms in the genes encoding these putative transporters may affect transport specificity and, hence, antigen presentation.

Regulation of ABC Transporters

It is not surprising that many ABC transporters are regulated at the level of synthesis and expressed in specific cell types, or in response to specific growth conditions. For example, many bacterial uptake systems are only expressed in the presence of their specific substrates. The activity of ABC transporters, once expressed, may also be regulated. For example, protein kinase-mediated phosphorylation regulates activity of the cystic fibrosis gene product CFTR (Cheng et al 1991). ABC sugar transporters in bacteria (e.g. for maltose, Nelson & Postma 1984) are inhibited by direct interaction between a component of glucose metabolism (Enzyme III) and the ABC transporter such that glucose is used as the preferred carbon source.

The above, while important for the cell, are unrelated to the general mechanisms by which ABC transporters function. More relevant, mechanistically, is the possibility that changes in the selectivity or specificity of ABC transporters could provide a point of control. The only known example is for certain bacterial uptake systems whose specificity can be varied by utilizing alternative periplasmic-binding proteins (such as the lysine-arginine-ornithine and histidine-binding proteins, which deliver different substrates to the same HisQMP transmembrane complex; Higgins & Ames 1981). Nevertheless, a number of other possibilities have been suggested. For example, the *ami* locus, which encodes the oligopeptide transporter of *Streptococcus*, includes an extra gene (*amiB*) that is not part of the core transporter (Alloing et al 1990). The *amiB* gene product resembles the ArsC protein, which is known to modulate the substrate specificity of the arsenate exporter (although the latter is not an

ABC transporter) (Chen et al 1986a); thus AmiB might also affect substrate selectivity. Another possible example is the oligopeptide transporter of *B. subtilis* (encoded by the SpoOK locus), which has two ATP-binding domains, OppD and OppF. Unlike the equivalent *S. typhimurium* system, OppF is not needed for peptide transport or bacterial sporulation: it is, however, required for competence and may therefore alter specificity or some other activity of the transporter (Perego et al 1991; Rudner et al 1991). The potential for such modulation of ABC transporters deserves attention in the future.

Regulation by ABC Transporters

ABC transporters can be adapted to transport specific regulatory molecules and, hence, regulate cell physiology. For example, the SpoOK locus of *B. subtilis* encodes an ABC transporter required for the initiation of sporulation, which probably mediates the uptake of a regulatory peptide (Perego et al 1991; Rudner et al 1991). The NodI protein of *Rhizobium* is required for the development of nodules (Evans & Downie 1986) and the HetA protein of *Anabaena* for differentiation into heterocysts (Holland & Wolk 1990), presumably by transporting a regulatory molecule into or out of the cell. As an alternative to transporting a regulatory molecule, the process of transport might itself have a regulatory function. Thus the PhoU protein is a peripheral membrane component encoded together with the phosphate transporter of *E. coli* (Surin et al 1985). PhoU is not required for transport, but regulates phosphate metabolism in the cell; it is suspected that PhoU interacts with components of the transporter, in some way sensing the transport event (rather than simply the presence of phosphate in the cell) and initiating its regulatory function. Doubtless, other regulatory events specific to individual ABC transporters and their cellular functions will be uncovered in the not too distant future.

Drug and Antibiotic Resistance

MICROORGANISMS Several examples of ABC transporters that mediate drug or antibiotic resistance by pumping the offending agent from the cell have been identified. Some are of considerable medical importance. For example, erythromycin resistance of *Staphylococcus* is mediated by the ABC protein MsrA (Ross et al 1990). The DrrAB and TlrC proteins of *Streptomyces* export daunomycin and tylosin from the cell, respectively, and thus confer resistance (Guilfoile & Hutchinson 1991; Rostek et al 1991). It should be noted, however, that not all cases of resistance by export involve ABC proteins; tetracycline resistance, for example, is mediated by a member of the twelve-membrane-helix family of transporters energized by the electrochemical gradient.

In eukaryotic microorganisms similar phenomena have been observed. Thus

the ltpgpA protein of *Leishmania* confers resistance to methotrexate and to heavy metals, presumably exporting them from the cytoplasm. Importantly from a medical standpoint, an ABC transporter, pfMDR, has been associated with chloroquine resistance in some strains of the malarial parasite *Plasmodium* (Foote et al 1989, 1990). Its precise role in resistance is still unclear. Mutations in the *pfdm1* gene may alter the specificity/activity of a normal cellular transporter such that it can recognize chloroquine and pump it from the cell (Foote et al 1990). Alternatively, pfMDR may affect chloroquine accumulation indirectly, for example by influencing the pH of food vacuoles in which chloroquine accumulates (Geary et al 1990).

P-GLYCOPROTEIN AND MULTIDRUG RESISTANCE OF CANCERS The development of resistance to multiple chemotherapeutic drugs is a major problem in cancer treatment and is responsible for hundreds of thousands of deaths worldwide. Overexpression of the 170 kd P-glycoprotein, a normal cellular component, is frequently associated with multidrug resistance. P-glycoprotein is an ABC transporter that pumps hydrophobic drugs out of cells in an ATP-dependent fashion, thereby reducing the cytoplasmic concentration of the drugs and hence their toxicity (comprehensively reviewed by Endicott & Ling 1989; Gottesman & Pastan 1988).

The P-glycoproteins are a small multigene family with two very closely related genes in humans and three in rodents (Endicott et al 1991; Table 1). Only one of these genes in humans, *MDR1*, confers drug resistance when overexpressed; the function of the other gene (*MDR3*) is unknown. The designations P-glycoprotein and multidrug resistance protein refer only to this small multigene family and its true homologues in other species. Even the DrrAB proteins of *Streptomyces*, which pump daunomycin and doxorubicin from cells (two P-glycoprotein substrates), are no more related to P-glycoprotein than are other ABC transporters with entirely different substrate specificities.

Unlike other ABC transporters, P-glycoprotein exhibits a relatively broad specificity: it can handle a wide range of chemically dissimilar drugs, yet shows little or no affinity for most normal cellular components. This broad specificity, of course, presents a major obstacle to the development of new chemotherapeutic drugs. It is also presents an investigative challenge. A mechanism to account for this unusual specificity has recently been proposed (Higgins & Gottesman 1992). It is suggested that substrate recognition is a two-step process. First, drug must intercalate into the lipid bilayer and only then can it be recognized by the transporter, which pumps it out of the bilayer. Thus the binding site on the protein can have a relatively broad specificity, yet only be available to those compounds that can intercalate appropriately

into the bilayer. This model is supported by evidence that P-glycoprotein substrates can interact with the protein from the lipid phase (e.g. Raviv et al 1990). Furthermore, P-glycoprotein can be labeled with hydrophobic agents such as forskolin and its derivatives; the more lipophilic the derivative the more efficiently it labels (Morris et al 1991). Since MDR drugs compete with forskolin for labeling, this implies that the substrate-binding site is accessible from the lipid phase. This "flippase" model not only provides an explanation for the specificity of P-glycoprotein, but has implications for the organization of the transmembrane domains and the mechanisms of solute translocation (Higgins & Gottesman 1992; see below).

While it is clear that P-glycoprotein can pump hydrophobic drugs from the cell, its normal cellular function is unclear. Many suggestions based on its tissue distribution include the expulsion of toxic metabolites from the cell and the export of steroid hormones or progesterone (e.g. Arceci et al 1988). Recent studies have shown, however, that expression of P-glycoprotein is associated with a volume-regulated chloride channel (Valverde et al 1992; Gill et al 1992; see below). There is now strong evidence that volume-regulated chloride channels with the same characteristics as the P-glycoprotein-associated channel play an important role in regulating epithelial cell volume. P-glycoprotein is appropriately located in epithelial apical membranes. Thus the regulation of cell volume through chloride channel activity may reflect a normal cellular function of P-glycoprotein (Valverde et al 1992). How this meshes with drug transport is unclear, although the chloride channel and drug transport functions are clearly distinct activities (Gill et al 1992; see below). Perhaps P-glycoprotein serves different functions in different cell types. Much remains to be learned about the cellular role(s) of this important protein.

Channel Functions: CFTR and P-glycoprotein

The product of the cystic fibrosis gene, CFTR, consists of the four core domains of an ABC transporter, together with an additional highly hydrophilic domain, the R (regulatory)-domain (Riordan et al 1989). It has long been known that one of the major biochemical defects in cystic fibrosis cells is altered epithelial chloride channel activity (reviewed by Welsh 1990). Since the identification of the CF gene, strong evidence that CFTR functions as a cAMP-regulated chloride channel has been presented. Thus expression of CFTR in heterologous cells generates channel activity (Anderson et al 1991a; Kartner et al 1991), and mutation of the transmembrane regions of CFTR alters the ion selectivity of this channel (Anderson et al 1991b). Furthermore, CFTR has been reconstituted into a lipid bilayer and shown to generate a channel (Bear et al 1992). The R-domain plays a role in opening the channel in response to cAMP and PKA (Cheng et al 1991; Rich et al 1991). It must

be emphasized that the CFTR-mediated movement of chloride across the membrane is channel-like and is not simply ATP-dependent chloride transport. (The transporter-channel distinction is discussed below.)

Recent studies on the multidrug resistance P-glycoprotein show it too is associated with chloride channel activity, although the P-glycoprotein-associated channel is regulated by cell volume rather than by cAMP (Valverde et al 1992; Gill et al 1992). Thus P-glycoprotein appears to be bifunctional, associated with both drug transport and chloride channel functions. This, of course, raises the possibility that CFTR may also be bifunctional, with a transport function yet to be identified. It is important to stress that the transport and channel functions associated with P-glycoprotein are distinct and separable; it is not a case, for example, of cotransport of drugs and chloride (Gill et al 1992). Indeed, drug transport requires ATP hydrolysis, while the channel function can be supported by non-hydrolyzable analogues (Gill et al 1992). It is interesting to consider whether drugs and chloride pass across the membrane via the same translocation pathway, particularly given the very different chemical characteristics of cationic hydrophobic drugs and chloride ions. An alternative possibility is that P-glycoprotein interconverts between channel and transporter states with different transmembrane pathways (e.g. a monomer-dimer transition; see below). Clearly, much remains to be learned. It should also be emphasized that even though some ABC transporters have associated channel activities, this is not necessarily a function of all such transporters. This would prove a nonsense, for example, in bacterial cells that have large numbers of ABC transporters. Indeed, preliminary data show that several bacterial transporters do not normally generate channel activity.

The identification of channel activities associated with certain ABC proteins has important mechanistic implications, particularly for the role of ATP. ABC transporters couple ATP hydrolysis, stoichiometrically, to the active translocation of solute across the membrane (see above). Channels do not, however, normally require an energy source. Yet, ATP is required for both CFTR and P-glycoprotein-associated channel functions (Anderson et al 1991c; Gill et al 1992). Presumably ATP plays a gating role, opening and closing the channel. The CFTR channel is reported to require ATP hydrolysis while the P-glycoprotein-associated channel requires binding, but not hydrolysis. Whether the two differ mechanistically awaits a more complete understanding of the molecular basis of channel activation.

MECHANISMS OF SOLUTE TRANSLOCATION

The absence of any structural information on the nature of the ABC transport complex, or indeed of any active transporter, ensures that our understanding of the molecular basis of solute translocation is superficial. Nevertheless, it

is worth considering a few points, only if to eliminate certain models and provide others to be shot down.

Structure of the Transmembrane Complex

Transporters are generally considered to bind their substrate in an enzyme-like fashion and to undergo an energy-dependent conformational change, which releases the substrate on the opposite face of the membrane (see below). Early models implying a major reorientation of the transport protein within the membrane (e.g. a rotating carrier) are clearly incorrect on thermodynamic grounds. More likely, a small conformational change realigns the substrate binding site within a pore-like translocation pathway formed by the transmembrane domains of the transporter (Figure 5A). The term channel is sometimes used to describe such a structure, although as this term has mechanistic connotations (see below), it may cause confusion if used to describe structural features of the pathway through which solute crosses the membrane. The term pore-like is used with no mechanistic implications and could equally well apply to the structure of the transmembrane domains of a channel or transporter. The notion of a pore-like structure is supported by the observation that ABC transporters can handle polypeptides: these must be exported in an unfolded, elongated state and presumably span the membrane during the translocation process. Indeed, the microsomal protein translocator (although not an ABC transporter) allows the passage of ions once its protein substrate is removed, consistent with a pore-like translocation pathway (Simon & Blobel 1991).

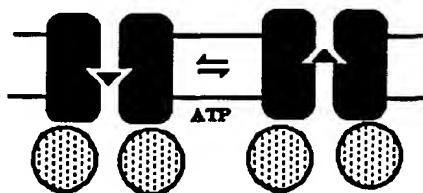
How is this pore-like transmembrane pathway organized? It must be based on the twelve transmembrane segments (helices?) of the transmembrane domains (although there have been suggestions that a loop extending from the ATP-binding domain might insert into this pore and hence contribute to the overall structure; Kerppola et al 1991; see above). It is interesting to note that other families of transporters also appear to be based on the principle of two-times-six transmembrane segments (Maloney 1990). Thus the twelve-transmembrane-helix family of transporters functions as monomers (Henderson et al 1991), and the anion translocators of mitochondria require two domains, each of six-transmembrane segments (Palmieri et al 1990). Does this requirement for twelve transmembrane segments reflect a fundamental organizational constraint?

A naive model places the twelve transmembrane segments into a pore that provides a hydrophilic pathway through which the substrate can pass (Figure 5B). However, this would require thermodynamically unstable helix-packing arrangements, does not take into account the pseudosymmetry between the two domains of six helices, and cannot readily accommodate those transporters with more or less than twelve transmembrane segments. More likely, the

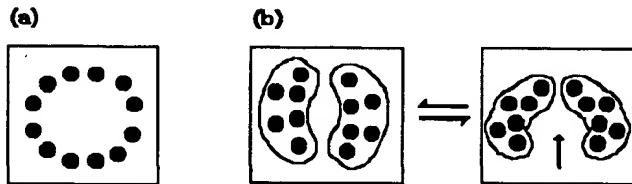
transmembrane helices are packed into two blocks with solute translocation occurring at the interface between these blocks (Figure 5B). However, no experimental data address this point directly.

It is generally assumed that the transmembrane segments provide a discreet microenvironment within the membrane (i.e. a hydrophilic hole) through which the substrate passes without ever coming into contact with the lipids

A.



B.



C.

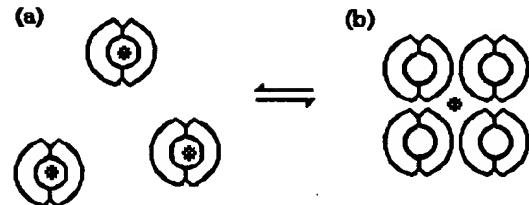


Figure 5 Models. (A) Reorientation of a substrate-binding site within the transmembrane regions of a transporter. Note that the binding site does not have to be in the middle of the bilayer as depicted here. Indeed, for ABC transporters it may be towards the cytoplasmic face of the membrane as cytoplasmic loops can affect substrate specificity (see text). (B) Packing of transmembrane segments viewed from the membrane surface. Each dot represents a transmembrane segment. These might be organized as a single hole (a), or in two blocks of six with substrate being translocated along the interface between them (b). The possibility that "breathing" allows access to the transporter from the lipid phase also needs to be considered. (C) Transmembrane pathways. ABC transporters may function as monomers to provide a pathway for solute through the membrane (a). Oligomerization could provide an entirely different pathway (b). The pathway through the membrane is indicated by \bullet . This could provide for alternate pathways for chloride channel and drug transport activities of the apparently bifunctional P-glycoprotein (see text).

that make up the bilayer; the substrate is shielded from the hydrophobic environment. It should be noted, however, that the possibility that substrates pass across the membrane at a lipid-protein interface cannot formally be excluded. Furthermore, the assumption that the substrate and its binding site are shielded from the lipid phase has recently been challenged (Higgins & Gottesman 1992). Observations with the multidrug resistance P-glycoprotein have led to the suggestion that substrate can gain access to the core of the transporter from the lipid phase (see above). This has general implications for the organization of the transmembrane domains. These data imply that the transporter may open in two planes, perpendicular and parallel to the plane of the membrane, which allows substrate entry from either the aqueous or the lipid phases (Higgins & Gottesman 1992). This could be achieved by "breathing" of the transmembrane segments in the plane of the membrane (Figure 5B). This model is supported by the observation that exported proteins containing stop-transfer sequences can end up spanning the bilayer; they must be able to exit laterally from the translocation pathway directly into the lipid phase (Simon & Blobel 1991). Data with other transporters are also consistent with this model. Thus the lactose permease appears as a cleft rather than a hole in electron microscopy (Li & Tooth 1987; Costello et al 1984). The substrate-binding site of the glucose carrier can be labeled with lipophilic forskolin derivatives (Wadzinski et al 1991); although glucose is not lipid-soluble, the binding site appears to be available from the lipid phase. Similarly, the lactose permease binds dansylated sugars, and the affinity increases the more hydrophobic the labeling agent (Schuldiner et al 1977). Finally, many substrates for ABC transporters may be more hydrophobic than is generally recognized. The a-mating peptide transported by STE6 of yeast is farnesylated (Anderegg et al 1988), and hemolysin is modified by an acyl group prior to transport (Hardie et al 1991; Issartel et al 1991). Although not required for the transport of these polypeptides, the lipid group may improve kinetics, even if it simply restricts diffusion to two dimensions (i.e. in the plane of the membrane) rather than three.

Channels and Transporters

The great majority of ABC transport systems mediate active transport. However, two apparently typical ABC transporters, CFTR and P-glycoprotein, have recently been associated with ion channel functions (see above). What, if anything, is the difference between a channel and a transporter? In the absence of detailed structural and mechanistic data, any distinction can only be operational, based on indirect experimental observations. Transporters are generally considered to be enzyme-like, interacting stoichiometrically with their substrate and undergoing defined conformational changes during each transport cycle. In contrast, channels are more akin to holes which, when

open, allow non-stoichiometric passage to molecules with appropriate characteristics. This distinction is based on several types of experimental observation. First, kinetic and biochemical characterization of several transporters have revealed enzyme-like intermediate states during each transport cycle, often interpreted as the alternate exposure of a substrate-binding site at each face of the membrane (reviewed by Stein 1990). Second, many transporters have been shown to be stoichiometrically coupled to other events (such as ATP hydrolysis or proton movement). Third, the turnover number of transporters (i.e. the number of molecules of substrate that can be transported per second) is necessarily restricted by enzyme-like conformational changes. In contrast, channels are ultimately diffusion-limited, and their turnover number can be several orders of magnitude greater than has ever been measured for a transporter. Finally, channels simply facilitate equilibration of substrate in response to concentration and electrochemical gradients. Transporters, in contrast, can utilize energy (ATP in the case of ABC transporters) to concentrate substrate against a gradient.

The finding that ABC transporters can be associated with both channel and transport functions raises the question of how similar proteins can function as transporters, channels, or both? It should be noted that, at least superficially, the transmembrane domains of CFTR and P-glycoprotein appear rather different from those of other known channels (Higgins & Hyde 1991). A key question is whether the organization of the transmembrane domains is the same for an ABC transporter, which functions as a transporter, as for one that functions as a channel? At one extreme, the translocation pathway formed by the transmembrane domains of ABC transporters and channels may be very similar: a small structural change might open the transmembrane pathway of a transporter and allow it to function as a channel. At the other extreme, the transmembrane pathways may be very different. For example, transport activity might be associated with a monomeric protein, while channel function might involve a very different transmembrane translocation pathway generated by association of the protein into di- or oligomers (Figure 5C). Answers to such questions are keenly awaited.

Energy Coupling and/or Gating

Assuming that the transmembrane domains of an ABC transporter generate a pore-like structure that forms the pathway for solute translocation across the bilayer, how is this pathway opened and closed? At one extreme the transmembrane domains might simply form a hole: the ATP-binding domains could serve to open and close this hole. This would be analogous to a gated channel and may be relevant to the function of the CFTR and P-glycoprotein-associated channels. However, the transmembrane domains do not simply

form an open channel in the absence of the ATP-binding domains. Furthermore, most ABC transporters are more than gated channels: solute can be concentrated against a gradient, and they show directionality and stoichiometry. The ATP-binding domain must, therefore, play a more positive role. Models in which the ATP-binding domains pump substrate into the pore-like transmembrane structure could be envisaged. They do not, however, accord with the fact that the transmembrane domains play a large part in determining substrate specificity (see above). Furthermore, at least for P-glycoprotein, substrate can gain access to its binding site on the transmembrane domains even in the absence of ATP. Indeed, the interaction of substrate with the transmembrane domains appears to stimulate ATP hydrolysis, not vice versa. Thus ATP hydrolysis must act at a step subsequent to substrate binding. Classical kinetic analyses of transporters imply a conformational change exposing substrate-binding sites to opposing sides of the membrane. The most appealing model then, consistent with the available data, is that substrate interacts with its binding site located within a pore-like structure: ATP hydrolysis reorientates this site to expose it to the opposite face of the membrane (Figure 5A). However, it is equally possible that reorientation of the binding site is facilitated by substrate-binding and that ATP hydrolysis resets the orientation in order to impose directionality and the ability to actively accumulate substrate.

CONCLUDING REMARKS

There is now a substantial body of knowledge pertaining to ABC transporters and their cellular and physiological roles. In the past two or three years, their fundamental importance for many cellular processes has been established. Over forty ABC transporters are known in bacteria. It is not unreasonable to expect that an equally large number, handling a wide variety of substrates and with equally novel and varied cellular roles, remain to be characterized in yeasts and mammalian cells: the known transporters may only reflect the tip of an iceberg. A rather complete description of ABC transporters and the basic mechanisms by which they function has now been derived. Refinements to these working models will doubtless be made as further data accrue. Nevertheless, a quantum leap is necessary to substantially increase our understanding of the molecular mechanisms of transport: this can only come from structural determinations. The elucidation of the molecular basis of transmembrane transport remains a considerable challenge for the future.

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